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CLINICAL AND EXPERIMENTAL STUDIES WITH THE CITROVORUM FACTOR

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CLINICAL AND EXPERIMENTAL STUDIES WITH THE CITROVORUM FACTOR

By RUTH WOODS

The existence of "folinic acid", or the citrovorum factor was discovered in 1948 when Sauberlich and Baumann reported that the microorganism L. *citrovorum* required for normal growth an unidentified factor in liver in addition to the standard synthetic nutrient media usually found satisfactory for similar microorganisms. Between then and the end of 1950, workers in various independent laboratories confirmed the existence of this new factor, established its status as a derivative of folic acid, created it in synthetic form and finally, isolated pure crystals of the synthetic material. Its biological behavior and its chemical properties were investigated also, and by the beginning of 1951 it became apparent that the new citrovorum factor has potential significance for man (as well as for other animal species; e.g. the rat, mouse, and chick). All of these findings have been described in detail in an earlier issue of this Review (1).

Present indications are that the citrovorum factor may be of considerable value in the clinical treatment of the anemias (including pernicious anemia, nutritional anemia, and the anemia of sprue) as well as in the clinical management of the leukemias, indirectly, as an adjunct to the therapy of these conditions with the various folic acid antagonists.

This paper will review some of the newest work on the use of citrovorum factor in the treatment of anemia and leukemia in human subjects.

Relation of Citrovorum Factor to Other Anti-Pernicious Anemia Factors:

In order to better understand the rationale behind the use of the citrovorum factor (CF) in the anemias and in offsetting the symptoms of toxicity resulting from the use of folic acid antagonists in leukemia, a brief summary of the findings presented in a previous Review (1) on the citrovorum factor follows:

CF is an essential nutrient for the organism, L. *citrovorum*. In this capacity it can be replaced to some extent by thymidine, or by very large amounts of folic acid, but not by vitamin B_{12}. Moreover, CF can replace folic acid as a growth factor in all other organisms thus far tested. When CF concentrates are subjected to acid hydrolysis, all activity characteristic of the citrovorum factor is lost, but folic acid activity remains unchanged. The citrovorum factor is derived from liver or yeast extracts and can also be isolated

from the urine of both rats and man given supplementary folic acid. Further, it has been reported that CF can be synthesized biologically by rat liver slices, a process which is considerably enhanced by the presence of extra folic acid and ascorbic acid. CF, or rather folinic acid (believed to be identical with CF), has been synthesized chemically by the hydrogenation of formylfolic acid, a functional derivative of folic acid.

In addition to possessing folic acid growth-promoting activity, often superior to that of folic acid itself, experiments with folic acid antagonists revealed that CF competitively inhibits the growth-depressing effects of these compounds (in L. citrovorum, S. faecalis, mice, chick embryos, and in mouse leukemia). Folic acid gave little or no protection against its own antimetabolites; vitamin B_{12} was completely ineffective; thymidine was required in extremely large dosage, the effect, therefore, being judged to be one of a non-competitive type.

CF in Clinical Treatment of Pernicious Anemia:

Because of these intriguing relationships among CF, folic acid, and thymidine, as well as because of the relation of the latter two to the treatment of anemia, studies were begun to determine the possible effect of CF when administered to patients with various types of anemias.

Probably the first of such studies to be reported were those of Spies and associates (2) at the Nutrition Clinic in Birmingham, published in December, 1950. In these studies, both folinic acid and citrovorum factor were given to a group of patients having pernicious anemia, nutritional macrocytic anemia or the macrocytic anemia of sprue.

Unit Equivalents of Folinic Acid and CF:

Folinic acid was supplied in 1 cc. ampules containing 100 units. One unit of folinic acid (Lilly) is equivalent approximately to one microgram of pure citrovorum factor, which is the same as 10 Lederle units or 4,900 Baumann-Sauberlich units.

The citrovorum factor (Lederle) was also provided in 1 cc. ampules—these contained 20 million CF units. One unit is equivalent to 0.0001 micrograms of folic acid—that is, 10 million units equal 1 mg. of folic acid.

Since only very small amounts of folinic acid were available for study, patients were given only enough to determine if it had any anti-anemic properties. Citrovorum factor, being available in large amounts, was given in large doses to see, not only if it had anti-anemic properties, but if a maximal response could be obtained.

Six patients were treated with folinic acid and four with citrovorum factor. Of the former group, only two showed a response (Fig. 1) — one

with pernicious anemia, the other with sprue. As was to be expected, the responses were slow and submaximal as contrasted to previous therapy with folic acid or vitamin B_{12}. Nevertheless, both clinical* and hematopoietic improvement was obtained.

The four patients who were treated with citrovorum factor all received fairly large doses and therefore all responded. Fig. 2 illustrates typical responses obtained with citrovorum factor in a case of pernicious anemia.

As can be seen, there was a prompt hematopoietic response. Reticulocytosis began to increase by the third day, reaching a peak on the eighth day of therapy. Both the hemoglobin content and red blood cell count of the blood showed a rapid increase, reaching good levels by the end of two weeks of treatment. Clinical responses paralleled the hematopoietic progress and consisted of returning strength and vigor, improvement of tongue, disappearance of abnormal gastrointestinal symptoms of sprue.

From these observations, Spies *et al* concluded that citrovorum factor (and probably folinic acid as well, if provided in adequate amounts), administered intramuscularly, is an effective anti-anemic substance in promoting blood regeneration in pernicious anemia and other allied types of anemias.

Simultaneously, Meyer, Brahin and Sawitsky (3) described similar studies on the use of CF in pernicious anemia patients. Although the dosages they used were of a similar order to those used by Spies' group, their results appeared far less satisfactory. Their study included five patients with pernicious anemia in relapse, all with megaloblastic† bone marrow. In general, they found that the daily administration, intramuscularly, of from 10 to 20 million units (1.5 to 3.0 mg.) of CF produced even after 6-7 weeks only submaximal reticulocyte response and suboptimal levels of hemoglobin and red blood cells. Maximum reticulocyte response was observed in one case only. Clinical improvement in appetite, strength and general well-being, however, was good in all cases, but did not approach the dramatic responses usually obtained with liver extract, folic acid or vitamin B_{12}. Neurological symptoms were improved in only one case, remaining unchanged in the other four.

The following month Ellison *et al* (4) also reported a study of the effect of citrovorum factor in pernicious anemia patients. In order to further explore the possible mechanism of its action, they tested the use of CF in these patients with, and without, the simultaneous administration of the CF and folic acid inhibitor, aminopterin.

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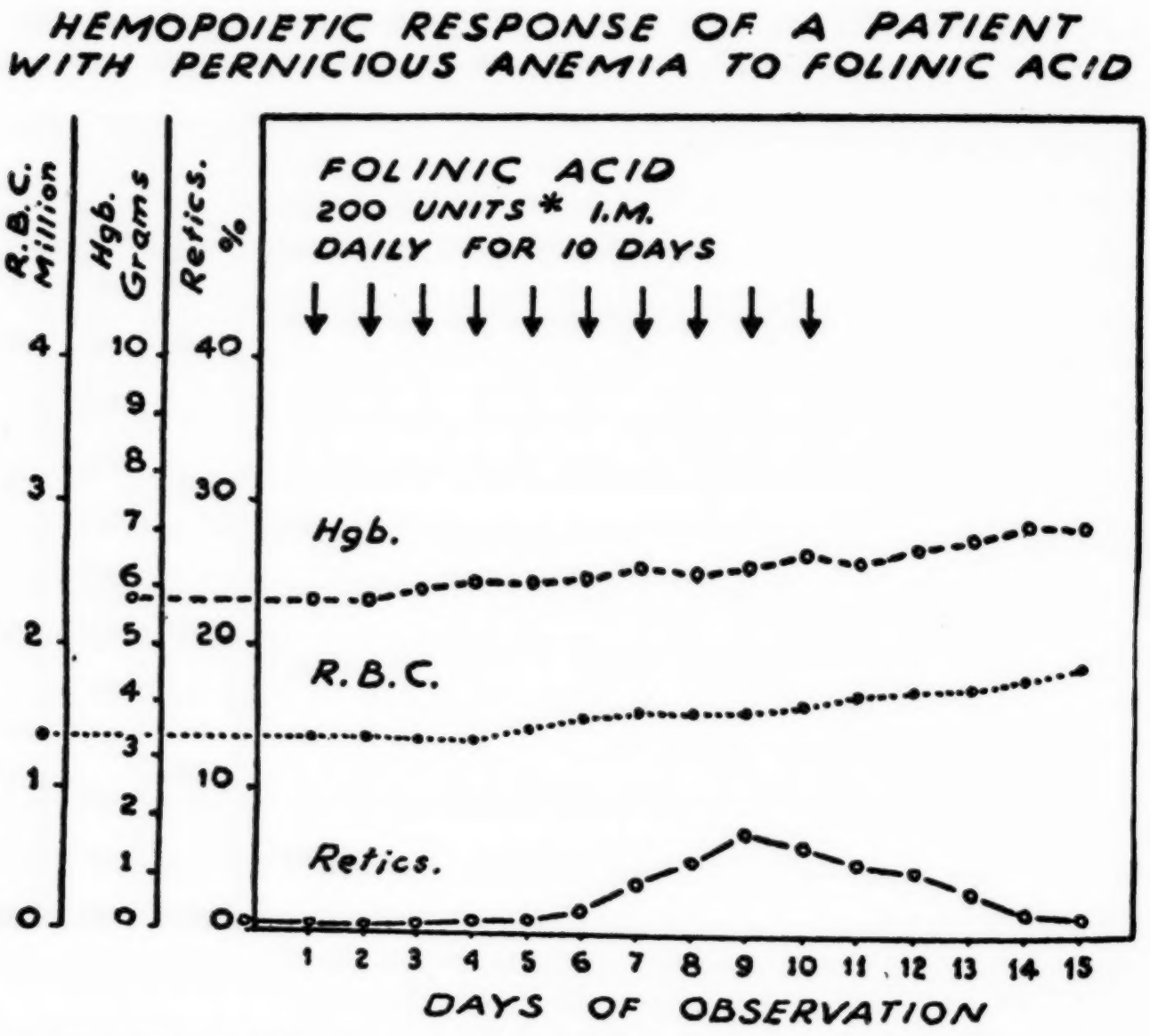
*Recovery from weakness, appetite improvement, clearing of fiery red tongue, slight recovery from edema of ankles and feet, improvement in bowel activity in sprue.

†A megaloblast is a large nucleated red blood cell—a primitive form of the erythroblast which is an early-developing form (hemoglobin-free) of the normal red blood cell


HEMOPOIETIC RESPONSE OF A PATIENT WITH PERNICIOUS ANEMIA TO FOLINIC ACID
R.B.C. Million
Hgb. Grams
Retics. %
FOLINIC ACID
200 UNITS * I.M.
DAILY FOR 10 DAYS
Hgb.
R.B.C.
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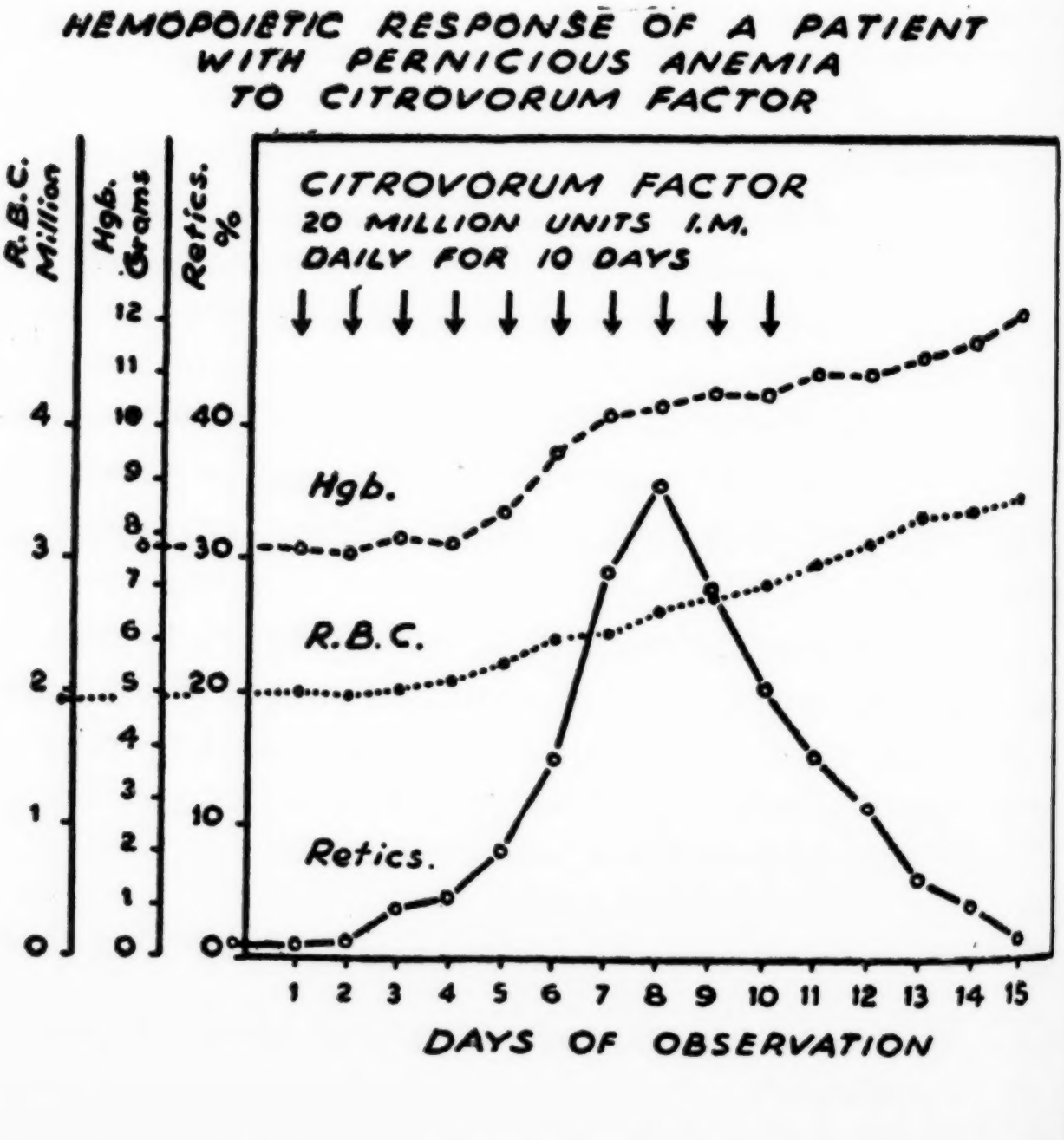
DAYS OF OBSERVATION
* 1 UNIT IS APPROXIMATELY 1. MICROGRAM OF PURE CITROVORUM FACTOR OR 10 LEDERLE UNITS

FIG. 1


HEMOPOIETIC RESPONSE OF A PATIENT WITH PERNICIOUS ANEMIA TO CITROVORUM FACTOR
R.B.C. Million
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CITROVORUM FACTOR
20 MILLION UNITS I.M.
DAILY FOR 10 DAYS
Hgb.
R.B.C.
Retics.
DAYS OF OBSERVATION

FIG. 2

Their subjects included 10 patients, all with Addisonian pernicious anemia associated with typical megaloblastic bone marrow. Of this group, six were treated with CF alone. As can be seen from the table (Table 1), only one of the first five had a maximal reticulocyte response. This patient received 10 million units daily (intramuscularly). Case 4, on the same dosage, had submaximal reticulocytosis and submaximal red blood cell increase after three weeks. Cases 1, 2 and 3, however, receiving only 5 million units CF daily, all had maximal red cell rises at 3 weeks, despite submaximal reticulocyte peaks. The abnormal megaloblastic bone marrow was converted to a normal, normoblastic marrow in all 5 patients. All of these first five patients also showed satisfactory clinical improvement—less dramatic than that occurring with B_{12} or liver extract therapy—but no change in neurological symptoms. These latter observations closely paralleled the observations of Meyer *et al.* The sixth patient in this group failed to show any type of response to 10 million units of CF daily, but subsequently improved on a single large injection of 120 mcgm. of B_{12}.

Table 1. Citrovorum Factor: Hematological Response in Patients with Addisonian Pernicious Anemia (4)

Case No.	1	2	3	4	5	6*
Citrovorum factor, mill. units I.M./day	5	5	5	10	10	10
Initial RBC, mill./cu.mm.	1.46	2.55	2.19	1.30	2.65	1.75
RBC, mill./cu.mm. at 3 weeks	3.09	3.56	3.27	2.51	3.75	
Expected RBC at 3 wks, mill./cu.mm.	3.15	3.69	3.54	3.04	3.76	
Day of reticulocyte peak	10	10	11	8	7	None
Max. reticulocytes, %	12.7	5.4	6.6	6.9	9.8	
Expected max. reticulocytes, %	24.5	10.0	16.0	27.0	9.0	20.5

*Subsequently received 120 mcgm. B_{12} I.M. and responded with reticulocyte and RBC rise.

Studies with CF and Aminopterin in Anemia: Ellison and co-workers then selected four additional patients (Cases 7, 8, 9, 10) to whom aminopterin was given in addition to specific therapy with folic acid and vitamin B_{12}. This was done in an attempt to inhibit their hematopoietic response, so that subsequent administration of CF could be studied for its possible effect in counteracting the inhibition caused by aminopterin.

Case 7 received 0.125 mg. aminopterin and 7 mg. folic acid on alternate days for ten days. (This amount of folic acid when given alone usually produces optimal hematopoietic response in pernicious anemia). There was a suboptimal rise of 10.9%. In a second ten-day period, 20 million units of citrovorum factor were substituted every other day for the folic acid during aminopterin administration. The patient showed a secondary reticulocyte peak of 6.1% indicating a response to CF.

Case 8 received twice the amount of aminopterin (0.25 mg.) and the same amount of folic acid (7 mg.) every other day for ten days. In this case, the folic acid was unable to overcome the inhibition of hematopoiesis caused by the larger dose of aminopterin, as evidenced by a complete absence of reticulocyte response. When CF replaced folic acid in a subsequent ten-day period, a partial reversal of aminopterin was achieved as indicated by a submaximal reticulocyte peak of 6.9% reached on the ninth day.

Cases 9 and 10 were given vitamin B_{12} simultaneously with aminopterin. The first of these also received folic acid, the three substances being given together on alternate days for ten days in the following amounts: aminopterin, 0.25 mg.; vitamin B_{12}, 30 mcgm.; folic acid, 15 mg. The other received only B_{12} and aminopterin (10 mcgm. and 1 mg., respectively), on alternate days for ten days. Both of these patients showed maximal reticulocyte response, therefore it was not possible to test the effect of CF.

The reversal of the effects of aminopterin by CF and the superiority of CF to folic acid in achieving this result coincides with similar reports for the action of CF in microorganisms (5, 6, 7, 8) in mice (5, 8, 9) and in mouse leukemia (10).

It was of interest to note that aminopterin had no inhibitory effect on the response of the patients given vitamin B_{12}. Although this would not seem to be surprising because of the probable difference in roles between folic acid vitamin B_{12} as they influence blood regeneration, it is in contrast to the findings of Meyer *et al* (11). This may be due to a relatively insufficient amount of aminopterin, as Ellison *et al* suggest, or to a possible difference in the nature of the effects produced by aminopterin and those produced by pernicious anemia. Finally, as already suggested, it may also be due to a difference in the mode of operation of folic acid and of vitamin B_{12}.

In contrast to these general findings, a recent paper by Jarrold and associates (22) indicates that not only is CF a potent hematological agent in cases of pernicious anemia in relapse, but that its potency is equivalent to —but not greater than—that of folic acid. They deduced further that CF, like folic acid (23, 24, 25) must be altered somewhere in the body before it can become active as a hematopoietic agent.

From the few preliminary studies made thus far, it would appear that the citrovorum factor may be of some value in the treatment of pernicious anemia if given in adequate amounts. Its behavior seems to be comparable to the action of folic acid, although its effect, as reported by the majority of workers, seems to be considerably less than that of either folic acid or B_{12}. This is somewhat disappointing in view of the greater growth-promoting activity of CF over folic acid in certain microorganisms which suggests that

CF may be the enzymatically active form of folic acid. The results with human subjects may possibly reflect effects due to a species difference.

CF in Clinical Treatment of Neoplastic Disease:

Role of Folic Acid Antagonists: The folic acid antagonists, aminopterin (4-amino folic acid) and amethopterin (4-amino-N^{10}-methyl folic acid) have been used for the past few years in the clinical treatment of certain types of leukemia and other neoplastic diseases (12, 13, 14). In general, although the findings have not always been consistent, these agents have been successful in arresting the neoplastic processes, although no permanent cures have been obtained. The chief drawback in the use of these potentially valuable anti-neoplastic agents has been the toxic side effects encountered. These toxic effects have greatly hampered their use because of the dangers of the folic acid deficiency induced. The toxic reactions have included gastrointestinal cramps, diarrhea, hemorrhage, ulceration of the palate and oral mucous membranes, leukopenia which may progress to an aplastic anemia and alopecia, or loss of hair. Furthermore, the appearance of these toxic signs may be delayed and the cumulative toxic dose of the drug may be exceeded before signs of toxicity become evident. For this reason, the doses of these folic acid antagonists administered are often less than necessary for an adequate therapeutic effect or the administration of the drug may often have to be interrupted before satisfactory control of the disease can be had. Folic acid, liver extract or vitamin B_{12} have been completely unsuccessful in combating these disturbing toxic reactions.

Experimental studies with mice have shown that, under certain specific conditions, folic acid can prevent—but not reverse—both the toxic (15) and anti-neoplastic (16) effects of its antagonists. When folic acid was administered simultaneously with aminopterin, or shortly thereafter, however, no protection from fatal toxic effects could be obtained (15).

Studies with Citrovorum Factor: When it became known that the newly discovered citrovorum factor is more effective than folic acid itself in preventing aminopterin toxicity in rats and mice (17), Greenspan and associates (18) decided to investigate further the protective possibilities of the new factor. They found that, whereas folic acid was completely ineffective, citrovorum factor completely protected mice against toxic effects of aminopterin when both were given simultaneously or even when the citrovorum factor was given *subsequent to* a lethal dose of aminopterin.

On the basis of these encouraging results, Schoenbach, Greenspan and Colsky (19) administered citrovorum factor to two patients with neoplastic disease being treated with amethopterin and aminopterin.

(Case 1): The first patient was treated for cancer of the lung* with ten mg. of aminopterin daily. After nine days beginning signs of mouth ulceration developed and within two weeks the white blood cell count had dropped to less than half of normal. After 18 days of continued anti-folic acid therapy, the patient was treated with 40,000,000 units of CF daily (equivalent to 14 mg. folic acid) for one week. Improvement was noted on the third day after treatment with CF and complete healing of the ulcers, by the fifth day, despite the continued administration of the original daily dose of 10 mg. aminopterin. White blood cell count rose to near normal level by the sixth day. Ten days after CF supplements were discontinued, but daily aminopterin dosage maintained, mouth ulcers reappeared. The neoplastic lesions remained unaltered during aminopterin administration, either alone or in combination with CF.

(Case 2): The second patient was treated for neoplastic metastasis to the lungs from an original breast neoplasm. She had been treated successfully with small doses of aminopterin and remained symptom-free for three months. The reappearance of neoplastic symptoms was followed by further treatment with aminopterin, but this was soon followed by toxic manifestations similar to those of Case 1. Folic acid even in large doses failed to counteract the aminopterin toxicity which became progressively worse, advancing to loss of appetite, severe pain on swallowing and loss of hair. The administration of CF was begun, in conjunction with continued aminopterin†, but was found ineffective when given at levels of 20,000,000 units CF per day. Three days after this dosage was increased to 40,000,000, improvement was noted, white blood cell count rose to normal, and complete healing of the mouth ulcers followed three days later. When CF was discontinued and aminopterin maintained, toxic signs reappeared in ten days.

Experimental Studies on Prevention of Anti-Folacin Toxicity by CF:

The preliminary human studies and early experimental studies on reversal of toxic effects of folacin antagonists by CF have been extended recently in an attempt to learn more about the mechanism of action, as well as about the range of dosages required under various circumstances.

Earlier studies had indicated the superior effectiveness of CF over folic acid and in counteracting toxicity of relatively high doses of aminopterin in mice (5). The next question was, would CF be effective against massive

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*Resulting from the spread (metastasis) of a neoplastic growth in the leg, previously amputated.

†Only 2.0 mg. aminopterin per day, as contrasted with 10 mg. amethopterin in Case 1.

dosages of folic acid inhibitors? Burchenal and Babcock (20) investigated this problem in mice given high acute (single) doses and high chronic (continuous daily) doses of A-methopterin. They found that considerable protection against an acute lethal dose could be achieved by as little as one-thirtieth that amount of citrovorum factor given either one hour before or one hour after the dose of antimetabolite. Almost complete protection from this dose of A-methopterin was given by doubling the amount of citrovorum factor given.

When the citrovorum factor was given 24 to 48 hours before the A-methopterin, however, no protective effect was noted. Similarly, there was little or no significant protective effect when CF was given four hours or more after the antimetabolite.

The fact that these amounts of citrovorum factor are capable of reversing the effects of A-methopterin, in contrast to folic acid which is ineffective against even small doses of A-methopterin, indicates, as these investigators point out, that aminopterin and A-methopterin are true antagonists of citrovorum factor rather than of folic acid. They remark, also, that since in previous studies of theirs (21), citrovorum factor prevented not only the toxic effects of A-methopterin in mice, but its anti-leukemic effect as well, they consider it unlikely that citrovorum factor may have any practical usefulness in the treatment of acute leukemia in children. This is somewhat unexpected in view of the positive findings of Schoenbach *et al* (19) with other types of neoplastic diseases. Continued research, however, will undoubtedly clarify this, as well as many other currently puzzling aspects of the biological significance of the new citrovorum factor.

Conclusion:

The discovery of the citrovorum factor (CF) just a few short years ago adds still another factor to the growing group of substances having specific anti-anemia properties—substances which include, among others, folic acid, ascorbic acid and vitamin B_{12}.

Whatever its potential practical use may be—either as a vitamin with activities closely resembling those of folic acid, or as an effective antagonist of anti-folic acid compounds—it is certain that this newest vitamin factor can be expected to contribute significantly to the disentanglement of the intricate interrelations which exist between itself and folic acid and vitamin B_{12}. When these interrelationships can be fully understood and appreciated, the mechanisms underlying the cause and cure of pernicious anemia, which has puzzled science since its recognition a century ago, will at last emerge from their decades-long obscurity to ultimate comprehension.

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NEWS DIGEST

DIET AND BLOOD CHOLESTEROL: Recent studies have implicated cholesterol as a causative factor in the formation of arteriosclerotic lesions. This has led to considerable speculation as to the dietary significance of this substance. Studies on natives of Umnak, Atka and Atter Islands in the Aleutians were reported some months ago which confirm the current theory that faulty cholesterol metabolism — rather than the actual cholesterol content of the diet — is the ultimate predisposing factor in arteriosclerosis.

The Aleutians, living almost exclusively on lean meat and fish, habitually consume a low-calorie, low-cholesterol, high-protein diet. Yet analyses showed that serum cholesterol levels of these people did not differ significantly from those of Americans who commonly eat a butter, egg, cheese and milk diet, often abundant in calories. Blood cholesterol levels were similar in both sexes, and there seemed to be a marked rise in serum cholesterol levels for both sexes following cessation of growth. The cholesterol ester: total cholesterol ratio was similar in both Aleuts and Americans. A small number of cases of hypercholesterolemia (high blood levels of cholesterol) were found. The authors conclude that the evidence suggests that endogenous mechanisms — i.e., the way in which the body handles cholesterol — are responsible for the level of cholesterol in the blood.—*Canad. Med. Assoc. J. 64,* 338 (1951).

DENTAL CARIES: A new method for anticipating and preventing tooth decay has been reported. The test involves measuring the protein content of the saliva. The level of salivary protein is said to give an indication of incipient tooth decay and of the general condition of the body. High levels of protein in the saliva constitute a danger signal. Persons with high protein saliva are advised to make dietary changes which include more milk, cod liver oil, vegetables, eggs and dark bread. More rest and fresh air are additional recommendations. Clinical studies conducted in New York City have shown a measurable decrease in the incidence of tooth decay as a result of these measures applied to potential caries victims, as determined by the salivary test. — *Scientific Amer. 184,* No. 2, 32 (1951).

VITAMINS IN LETTUCE: Recent home economics reports point out that lettuce, often the neglected background to a salad, is nutritionally valuable in its own right. It is rich in vitamins, especially

vitamin A—the outer leaves often possessing 20 times the vitamin A content of the paler inner leaves. Lettuce is also a fair source of ascorbic acid and of the B vitamins, riboflavin and folacin. Small amounts of vitamin E and vitamin K are provided by lettuce. This green is also an excellent source of essential minerals, particularly iron, copper, sodium and manganese. It is quite low in protein, but such protein as it does have, is of good quality. Lettuce has the added advantage of providing these nutrients along with a minimum of calories and is thus an important part of all types of low-calorie diets.—*Sci. News Letter,* May 12, 1951.

VITAMIN A AND MINERAL OIL: It is well known that mineral oil which is not utilized by the body may cause serious loss of fat-soluble vitamins from the diet by causing them to become dissolved in the oil and excreted with it. A recent report indicates, however, that the plasma level of vitamin A will be maintained during the administration of mineral oil if the amount and time of consumption of the oil are properly regulated. When one ounce of the oil was taken at bedtime; or several hours after the last meal; or when one-half teaspoon was swallowed three times a day with meals, no vitamin A loss was noted over a four-week period. Giving one ounce of oil at noon or one teaspoon with meals, however, reduced blood plasma levels of vitamin A from about 47 to 37 mg. per 100 cc. — *Proc. Cent. Soc. Clin. Research 23,* 101 (1950).

CASEIN GROWTH FACTOR: Crude casein is believed to contain an unidentified substance which is indispensable to normal growth and reproduction in the rat. Purified casein, while effective in maintaining normal growth, reproduction and lactation, resulted in the birth of offspring with a high rate of mortality. Those offspring which did survive fail to achieve completely normal growth levels and exhibited some degree of reproductive failure. Those who were able to bear young, however, produced offspring which failed to survive after three weeks of life. The chief biochemical lesions observed were localized in the liver. Vitamin B_{12} proved ineffective in combating the deficiency and was, therefore, not the active factor in crude casein. Small quantities of whole cow's milk, however, completely prevented the mortality on the diet of purified casein. —*Science 113,* 179 (1951).